Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/CA05/000356

International filing date: 04 March 2005 (04.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/549,559
Filing date: 04 March 2004 (04.03.2004)

Date of receipt at the International Bureau: 26 May 2005 (26.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

PA 1298663

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 25, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/549,559*
FILING DATE: *March 04, 2004*

CA/05/356

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. K. HAWKINS
Certifying Officer

16698 U.S.PTO

22859 60/549559 U.S.PTO 030404

PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
James	Russell	Vancouver, CANADA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Thrombomodulin (THBD) haplotypes predict outcome of patients

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number:

OR

☑ Firm or Individual Name	The University of British Columbia - Industry Liaison Office				
Address	#103 -6190 Agronomy Road				
Address					
City	Vancouver	State	BC	Zip	V6T 1Z3
Country	CANADA	Telephone	6048228594	Fax	6048225998

ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 17 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 13 ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees.

☐ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number:

☑ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

Date March 3/04

TYPED or PRINTED NAME Elaine Weiss

REGISTRATION NO. *(if appropriate)*

Docket Number:

TELEPHONE 604-875-4111 ext 68475

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Keith	Walley	Vancouver , CANADA

Number 2 of 2

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

TO:

Commissioner of Patents and Trademarks
US Patent and Trademark Office
2011 South Clark Place
Customer Window, Mail Stop Provisional Patent Application
Crystal Plaza Two, Lobby Room IB03
Arlington VA 22202

Enclosures:

1. Provisional application for patent cover sheet
2. Credit card payment for $80.00 filing fee
3. Specifications, 17 pages
4. Drawings, 13 pages

THE UNIVERSITY OF BRITISH COLUMBIA

March 4, 2004

Hon. Commissioner of Patents and Trademarks,
U.S. Patent and Trademark Office,
U.S. Department of Commerce,
Box Provisional Application,
Assistant Commissioner of Patents,
WASHINGTON, DC 20231,
U.S.A.

Dear Sir:

Re: Provisional Application for "Thrombomodulin (THBD) haplotypes predict outcome of patients."

UBC file no: 04-098

Enclosed please find the necessary documents for filing a Provisional Patent Application for the above-identified technology on behalf of The University of British Columbia. Also enclosed is Credit Card payment form PTO-2038 to cover the cost of the $80.00 application fee.

Thank you,

Sincerely,

Elaine A. Weiss, B.Sc, MBA
Technology Transfer Manager

Encl.

J·I·L·O

UNIVERSITY-INDUSTRY
LIAISON OFFICE

IRC Room 331
2194 Health Sciences Mall
Vancouver, BC, Canada V6T 1Z3

Tel: (604) 822-8580
Fax: (604) 822-8589
Web: www.uilo.ubc.ca

Title: Thrombomodulin (THBD) haplotypes predict outcome of patients.

Inventor: Keith Walley, Vancouver, CANADA
James Russell, Vancouver, CANADA

Abstract:

The invention involves characterization of polymorphisms in the Thrombomodulin (THBD) gene that are associated with adverse outcomes in patients. Methodologies for screening haplotypes are described. THBD haplotype screening will be useful in identifying patients who would benefit from increased monitoring by healthcare professionals, and/or possible therapeutic intervention, when said patient become subject to inflammation due to systemic inflammation response syndrome (SIRS), bacterial infection, bacteraemia, sepsis, septic shock, organ dysfunction, and trauma.

Background of the Invention:

Thrombomodulin (THBD) is a critical component of the activated protein C anti-coagulant pathway. THBD is a glycoprotein receptor found on endothelial cell surfaces that forms a high affinity complex with thrombin and inhibits its pro-coagulant activities (9-11). In addition, the thrombin-TM complex activates protein C (10), which binds to protein S on cell surfaces and degrades the clotting factors V and VIII (19, 26, 30, 31). THBD also has anti-inflammatory activity, inhibiting both cytokine formation and leukocyte-endothelial cell adhesion (7).

The activation of protein C by the THBD-thrombin complex is reduced in sepsis, resulting in perturbations in the coagulatory system and disseminated intravascular coagulation (22). THBD biosynthesis has been shown to be decreased by both endotoxin and hypoxia (28, 32). Microthrombi generated in this hyper-coagulable state lead to multiple system organ failure.

There are no known associations of polymorphisms of the THBD gene with outcome in sepsis. Animal studies suggest, however, that mutations in the THBD gene may be important in the pathophysiology of sepsis. A point mutation in THBD eliminated generation of activated protein C (APC) and inhibition of thrombin, generating a prothrombothic state in homozygous mutant mice (42). The THBD gene is well characterized and a number of polymorphisms have been examined for association with thrombosis or arteriosclerosis. A C-to-A polymorphism at position -133 and a G-to-A polymorphism at position -33 in the promoter region of the THBD gene cause decreased transcription of the TM gene, and consequently decreased expression of THBD (15, 29, 33). Both polymorphisms have been associated with myocardial infarction (MI), in

Caucasian and Asian populations respectively (15, 29, 33). A G-to-A polymorphism at position 125 results in the replacement of an alanine at amino acid 25 with a threonine, and is associated with a 2-fold increased risk of MI (8). Kunz et al. detected a novel missense mutation, Arg385Ser, in an elderly woman who had suffered 3 episodes of deep venous thrombosis (DVT) (21). The mutation was found to reduce THBD expression 2-fold and the co-factor activity of THBD 4-fold (21). It is possible that polymorphisms resulting in decreased expression of thrombomodulin on endothelial cells may contribute to a hyper-coagulable state in sepsis, and may be predictive of poor outcome.

Previous studies have tested single nucleotide polymorphisms (SNPs) in putative regulatory regions or rare SNPs found to cause missense mutations for association with thrombotic and atherosclerotic disease. Haplotypes are sets of SNPs in linkage disequilibrium with one another within a gene or segment of DNA that are inherited as a single unit (1, 43). Haplotypes serve as markers for all known and unknown SNPs within a haplotype, thus a haplotype-based approach to association studies can narrow down the search for a SNP that causes a change in phenotype (1). Haplotypes can be further grouped into sets of evolutionarily related groups, or clades (41). Haplotypes within a clade differ by only a few SNPs, and the variation within a clade is much smaller than the variation between clades. Grouping haplotypes into clades increases the statistical power to associate genetic variation with a change in phenotype (41). "Tag" SNPs (tSNPs) can be selected to uniquely define a clade and serve as markers for all SNPs within haplotypes of the clade.

Systemic inflammatory response syndrome (SIRS) is characterized by increased inflammation (relative to anti-inflammatory processes), increased coagulation (relative to anti-coagulant processes), and decreased fibrinolysis (5, 6, 17, 18, 24, 39). THBD is an endothelial cell surface receptor that binds circulating thrombin and inhibits its coagulant activities. The thrombomodulin:thrombin complex activates protein C and also has downstream anti-inflammatory effects. Polymorphisms in the THBD gene may disrupt anti-coagulatory and anti-inflammatory pathways, which may be associated to adverse outcomes in SIRS.

Previous literature reports a number of SNPs in the promoter region (G-201A, G-33A) and the coding region (F127A, C1418T, and G1456T) of the thrombomodulin gene have been tested for association to the occurrence and risk of thrombotic events and cardiovascular disease (8, 15, 21, 29, 33). The 33A allele has been found to decrease promoter activity of the thrombomodulin promoter region and may be associated with altered soluble thrombomodulin serum levels and coronary artery disease, carotid atherosclerosis, and myocardial infarction. The G-201A and G1456T polymorphisms were found to be rare in patients with severe thrombophilia and possibly functionally irrelevant. The

G127A polymorphism was weakly associated with increased risk of myocardial infarction in young men when additional risk factors such as smoking were present. The C1418T polymorphism may promote formation of varicose veins, and was associated with premature myocardial infarction and coronary heart disease. It was not associated with risk of venous thromboembolism. The associations of these polymorphisms with various thrombotic events and cardiovascular disease are uncertain and there have been a number of negative studies. There have been no previous studies examining the association of thrombomodulin polymorphisms with clinical outcome in critical illness, although the protein C pathway has been found to be central to the pathophysiology of sepsis.

Using a novel haplotype-based analysis, the inventors have identified single nucleotide polymorphisms (SNPs) in the THBD gene that identify a family of THBD haplotypes (clade) that are associated with statistically significant differences in important measures of clinical outcome such as survival and organ dysfunction. The present invention describes a better strategy of predicting patients who are at a greater risk of an adverse outcome, thus enabling earlier intervention and facilitating patient-tailored therapy based on genotype.

Summary of the Invention:

The present invention is concerned with single nucleotide polymorphisms (SNPs), which form haplotypes within the thrombomodulin (THBD) gene, which are predictive of patient outcome should that patient experience inflammation. Examples of inflammation experienced by patients include, but are not limited to, systemic inflammation response syndrome (SIRS), bacterial infection, bacteraemia, sepsis, septic shock, organ dysfunction, and trauma. This invention is novel, as the respective grouping of haplotypes described in the invention predict risk of inflammation and sepsis and patient outcome much more accurately than previously identified THBD polymorphisms.

In one aspect, the present invention provides the methodology required to screen patients in order to determine those at risk of an adverse outcome following inflammation. Genetic material is collected from the patient, most commonly by isolating leukocytes from the blood, but alternatively through a variety of biopsy methods, in order that the haplotype of the THBD gene can be ascertained. Determination of the haplotype from the genetic material can be done through a variety of methods commonly described in the art, including, but not limited to sequencing, restriction fragment length polymorphism (RFLP) analysis, hybridization, oligonucleotide ligation assay, ligation rolling circle amplification, allele specific PCR, and single base-pair extension assays.

Sequence data from any of the above mentioned assays could be stored in a database for future retrieval and haplotype analysis.

In another aspect of the invention, those patients at highest risk of inflammation are the infirm, elderly, and those individuals requiring hospitalization for a variety of reasons. These at risk individuals could be screened for the THBD haplotypes associated with elevated THBD such that those individuals can benefit from increased monitoring, and possible prophylactic treatments, in order to avoid the adverse effects of inflammation.

In another aspect of the invention, patients suffering from inflammation could be screened for the THBD haplotypes associated with decreased THBD such that those individuals can benefit from increased monitoring, and possible prophylactic treatments begun in order to avoid the adverse effects of inflammation.

In another aspect of the invention, the invention provides the methodology required to determine patient outcome following collection of genetic material and haplotype determination by analysing the THBD gene, whereby the specific THBD SNPs that form the respective haplotypes are located in the sequence described in SEQ ID NO:1.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material and haplotype determination by analysing the THBD gene, whereby 5 major haplotype clades could be defined by identifying the SNP at positions 5110, 5318 and 6235 of SEQ ID NO:1.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material by analyzing the THBD gene for 5110G/5318A/6235A, 5110A/5318A/6235A 5110A/5318A/6235G or 5110G/5318A/6235G haplotypes, whereby those individuals display an adverse outcome. This outcome is due to decreased survival arising from inflammation due to organ dysfunction, SIRS, sepsis, septic shock, bacterial infection, bacteraemia or trauma.

In another aspect, the invention further provides the methodology required to determine patient outcome following collection of genetic material by analysing the THBD gene at position for the 5110A/5318C/6235A haplotype, whereby those individuals do not display as severe an adverse outcome.

The sequence positions referred to in this invention and detailed in SEQ ID NO:1 refer to the sense strand of the THBD gene. It will be obvious to a person

skilled in the art that analysis could be conducted on the anti-sense strand to determine patient outcome.

The invention further provides for kits useful in carrying out the methods of the invention.

Brief Description of the Drawings:

Figure 1. Haplotype structure of the THBD gene in Caucasians. THBD haplotypes, inferred using PHASE from available data, are illustrated in the style of Patil et al. Each column represents a polymorphic site within the THBD gene and is labelled on the left with the position in the gene. Each row represents one of the inferred haplotypes ordered by phylogenetic relationship (Figure 2). MEGA II was used to sort haplotypes into clades separated by heavier lines. Haplotypes within each clade are very similar while clades differ substantially from each other. G5110A, A5318C, and A6235G were chosen as htSNPs.

Figure 2. Evolutionary relationships of THBD haplotypes. Haplotypes were sorted into 5 clades according to evolutionary tree structure. Clades are labelled by the alleles at 5110, 5318, and 6235. Tree branch distance is % difference between haplotype sequences. (Scale bar = 2% difference).

Figure 3. 28 day mortality rates by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades appeared to be associated with higher 28 day mortality rates than the A/C/A clade in 223 patients with SIRS.

Figure 4. 28 day mortality rates by THBD clade in patients with sepsis or septic shock on day one. The G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades showed a stronger association with increased 28 day mortality rates in 130 patients who had sepsis or septic shock on day one of the study.

Figure 5. 28 day mortality rates associated with G/A/A, A/A/A, G/A/G, and A/A/G clades vs A/C/A clade in 130 patients with sepsis or septic shock on day one.
(A) The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with significantly increased 28 day mortality rates than the A/C/A clade in patients who had sepsis or septic shock on day one of the study ($p=0.03$).
(B) Kaplan-Meier analysis of censored mortality data showed that the G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with greater mortality rates for the entire 28 day observation period ($p<0.03$).

Figure 6. DAF of cardiovascular dysfunction by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with fewer DAF of cardiovascular failure (p=0.02) and fewer DAF of vasopressors (p=0.03) in patients with sepsis or septic shock on day 1 of the study.

Figure 7. DAF of respiratory dysfunction by THBD clade. The G/A/A, A/A/A, G/A/G, and A/A/G clades were associated with fewer DAF of respiratory failure (p=0.02) and fewer DAF of ventilation (p=0.008) in patients with sepsis or septic shock on day 1 of the study.

Detailed Description of the Invention:

Definitions

Allele — One of the variant forms of a gene at a particular locus, or location, on a chromosome. Different alleles produce variation in inherited characteristics such as hair color or blood type. In an individual, one form of the allele (the dominant or major one) may be expressed more than another form (the recessive or minor one).

Clade — A group of haplotypes that are closely related phylogenetically. For example, if haplotypes are displayed on a phylogentic (evolutionary) tree a clade includes all haplotypes contained within the same branch.

Genetic Material — Genetic material refers to nucleic acids, whether deoxyribonucleic acid or ribonucleic acid, isolated from cells acquired from tissue or organisms.

Genotype — Genotype refers to the genetic makeup of an organism.

Haplotype — The set of genes, comprised of one allele of each gene, which make up the genotype.

Phenotype — Phenotype refers to the observable characteristics of an organism produced by the organism's genotype interacting with the environment.

Single Nucleotide Polymorphism (SNP) — A SNP is a place in the genetic code where DNA differs from one person to the next by a single nucleotide base pair. These slight genetic variations between human beings may predispose some people to disease and explain why some respond better to certain drugs.

Methods

Patient Cohort — All patients admitted to the Intensive Care Unit (ICU) of St. Paul's Hospital were screened for inclusion. This ICU is a mixed medical – surgical ICU in a tertiary care, university-affiliated teaching hospital of the University of British Columbia. SIRS was considered present and the patients included in the study when patients met at least two of four SIRS criteria. The SIRS criteria were 1) fever (>38 °C) or hypothermia (<35.5 °C), 2) tachycardia (>100 beats/min in the absence of beta blockers, 3) tachypnea (>20 breaths/min) or need for mechanical ventilation, and 4) leukocytosis (total leukocyte count > 11,000/μL) (2). Patients were included in this cohort on the calendar day on which the SIRS criteria were met. To decrease the confounding influence of population admixture secondary to ethnic diversity on associations between genotype and phenotype, only Caucasian patients were studied.

700 consecutive critically ill patients admitted to St. Paul's Hospital ICU were screened for inclusion into our study. Of these, 600 patients (94%) met the inclusion criteria of having at least two out of four SIRS criteria. From this group, 223 patients were Caucasian and were successfully genotyped and used as our final cohort for analysis.

Clinical Phenotype — Our primary outcome variable was 28 day mortality. Secondary outcome variables were measures of organ dysfunction and of the intensity of SIRS and sepsis.

Baseline demographics that were recorded included age, gender, medical or surgical diagnosis for admission (according to APACHE III diagnostic codes(18)), and admission APACHE II score. After meeting the inclusion criteria, data were recorded for each 24 hour period (8 am to 8 am) for 28 days to evaluate organ dysfunction, SIRS, sepsis, and septic shock.

Measures of organ dysfunction – Organ dysfunction for each organ system was defined as being present during a 24-hour period if there was evidence of at least moderate organ dysfunction using the Brussels criteria (Table 1) (36). Because data were not always available during each 24 hour period for each organ dysfunction variable, we used the "carry forward" assumption as defined previously (3). Briefly, for any 24 hour period in which there was no measurement of a variable, we carried forward the "present" or "absent" criteria from the previous 24 hour period. If any variable was never measured, it was assumed to be normal.

To further evaluate cardiovascular, respiratory, and renal function we also recorded, during each 24 hour period, vasopressor support, mechanical ventilation, and renal support, respectively. Vasopressor use was defined as dopamine > 5 μg/kg/min or any dose of norepinephrine, epinephrine, vasopressin, or phenylephrine. Mechanical ventilation was defined as need for

intubation and positive airway pressure (i.e. T- piece and mask ventilation were not considered ventilation). Renal support was defined as hemodialysis, peritoneal dialysis, or any continuous renal support mode (e.g. continuous veno-venous hemodialysis). In addition, the severity of respiratory dysfunction was assessed by measuring the occurrence of acute lung injury at the time of meeting the inclusion criteria. Acute lung injury was defined as having a PaO_2/FiO_2 ratio <300, diffuse infiltrates pattern on chest radiograph, and a CVP <18 mm Hg.

Measures of the intensity of SIRS and sepsis – Each of the four SIRS criteria were recorded as present or absent during each 24-hour period. Sepsis was defined as the presence of two or more SIRS criteria plus the presence of a known or suspected infection during the 24-hour period. Cultures that were judged to be positive due to contamination or colonization were excluded. Septic shock was defined as the presence of sepsis plus significant hypotension (systolic blood pressure <90 mm Hg or the need for vasopressors) during the same 24-hour period.

Days alive and free - To assess duration of organ dysfunction and to correct organ dysfunction scoring for deaths in the 28 day observation period, we calculated days alive and free of organ dysfunction (DAF) as previously reported (4). Briefly, during each 24-hour period for each variable, DAF was scored as 1 if the patient was alive and free of organ dysfunction (normal or mild organ dysfunction). DAF was scored as 0 if the patient had organ dysfunction (moderate, severe, or extreme) or was not alive during that 24-hour period. Each of the 28 days after meeting the inclusion criteria was scored in each patient in this fashion. Thus, the lowest score possible for each variable was zero and the highest score possible was 28. A low score is indicative of more organ dysfunction as there would be fewer days alive and free of organ dysfunction.

Microbiology – Microbiological cultures were taken for any patients who were suspected of having an infection. As this is a cohort of critically ill patients with SIRS, most patients had cultures taken. Positive cultures that were suspected of having been contaminated or colonized were excluded. Positive cultures that were deemed to clinically be clinically irrelevant were also excluded. Cultures were categorized as gram positive, gram negative, fungal or other. The sources of the cultures were respiratory, gastrointestinal, skin, soft tissues or wounds, genitourinary, or endovascular.

Haplotypes and Selection of htSNPs — Using unphased Caucasian genotypic data from the Coriell registry (frompga.mbt.washington.edu), we inferred haplotypes of THBD gene using PHASE software (40). We then used MEGA 2 to infer a phylogenetic tree to identify major haplotype clades (20). Haplotypes were

sorted into clades according to this phylogenetic tree and this haplotype structure was inspected to choose "haplotype tag" SNPs (htSNPs) (12, 16). We chose 3 ht SNPs that identified 5 major haplotype clades of THBD in Caucasians. The first SNP was a G-to-A transition at nucleotide 5110 relative to the start transcription site (rs1042580), the second SNP was an A-to-C transversion at nucleotide 5318 (rs3176123), and the third htSNP was an A-to-G transition at nucleotide 6235 relative to the start transcription site (rs1962) (NCBI Thrombomodulin accession number AF495471)(SEQ ID NO. 1). These SNPs were then genotyped in our patient cohort to define haplotypes and haplotye clades.

Blood collection and processing – The buffy coat was extracted from whole blood and samples transferred into 1.5 ml cryotubes and stored at –80°F. DNA was extracted from the buffy coat using the Qiagen DNA Blood Mini Kit. The genotypic analysis was performed in a blinded fashion, without clinical information.

Genotyping — Patients' genotypes at G5110A, A5318C and A6235G were determined by real time polymerase chain reaction (PCR) assay using specific fluorescence-labeled hybridization probes in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Inc.) as described by Livak (23). Briefly, the ABI Prism 7900HT uses a 5' Nuclease Assay in which an allele-specific probe labeled with a fluorogenic reporter dye and a fluorogenic quencher is included in the PCR reaction. The probe is cleaved by the 5' nuclease activity of Taq DNA polymerase if the probe target is being amplified, freeing the reporter dye and causing an increase in specific fluorescence intensity. Mismatched probes are not cleaved efficiently and thus do not contribute appreciably to the final fluorescent signal. An increase in a specific dye fluorescence indicates homozygosity for the dye-specific allele. An increase in both signals indicated heterozygosity. DNA from lymphocyte cell lines obtained from the Coriell Cell Repository was used to ensure the accuracy of the genotyping. The genotype of these cell lines at G5110A, A5218C and A6235 was determined using the ABI Prism 7900HT Sequence Detection system and compared to the genotype of the same cell lines determined by direct sequencing, given at *www.pga.mbt.washington.edu* (37).

Statistical Analysis – A cohort study design was used. A chi-squared test was used to test for an association between 28-day mortality and haplotype clades. This initial analysis identified the A/C/A haplotype clade as being distinct from all other clades. For subsequent analysis differences in clinical outcomes were compared between the A/C/A haplotype clade versus all other haplotypes combined. Rates of dichotomous outcomes (28-day mortality, sepsis and shock at onset of SIRS) were compared between the 2 groups of haploty[e clades · using a chi-squared test. Differences in continuous outcome variables between

the A/C/A haplotype clade and all other haplotype clades were tested using ANOVA. Baseline descriptive characteristics were compared using chi-squared test and ANOVA where appropriate. 28-day mortality was further compared between the A/C/A haplotype clade and all other haplotype clades while adjusting for other confounders (age, sec, and medical vs. surgical diagnosis) using a Cox regression analysis in addition to a Kaplan-Meier analysis. Haplotype clade relative risk was calculated. Genotype distributions were tested for Hardy-Weinberg equilibrium (14). We report the mean and 95% confidence intervals. Statistical significance was set at $p<0.05$. The data was analyzed using SPSS 11.5 for Windows and SigmaStat 3.0 software (SPSS Inc., Chicago, IL, 2003).

Discussion

We hypothesized that haplotype clades of thrombomodulin are associated with outcome from sepsis. To test this hypothesis we determined the haplotype structure of the thrombomodulin gene using publicly available data (from pga.mbt.washington.edu) (37). We then used cladistic analysis to group these haplotypes into related clades (Figures 1 and 2) (20) and subsequently determined a minimum set of "tag" SNPs (tSNPs) (Figure 1) that defined all 5 major haplotype clades of the thrombomodulin gene (12, 16, 41). We then tested for the association of these haplotype clades with 28 day mortality and organ dysfunction in a cohort of critically ill adults who had SIRS (35).

We found that the thrombomodulin haplotype clade defined by 5110A/5318C/6235A was significantly associated with decreased 28-day mortality ($p=0.03$), less organ dysfunction (cardiovascular, $p=0.02$; respiratory, $p=0.02$; hematologic system, $p=0.04$; neurologic, $p=0.02$, hepatic, $p=0.04$), and less sepsis ($p<0.02$) in patients who had sepsis or septic shock upon admission to the study. We conclude that the clades marked by 5110A/5318A/6235A, 5110A/5318A/6235A, 5110A/5318A/6235G, and 5110G/5318A/6235G are markers of adverse outcome in critically ill patients with sepsis or septic shock, and can be used to predict statistically significant differences in important measures of clinical outcome.

To our knowledge there are no other reported associations of polymorphisms of thrombomodulin with outcome in systemic inflammatory response syndrome and sepsis. A G-to-A polymorphism at position -33 in the promoter region of the thrombomodulin gene is particularly frequent in Asians, and is associated with coronary artery disease (CAD), myocardial infarction. The thrombomodulin G-33A polymorphism is near a consensus sequence for transcription control elements, and reporter gene assays have shown that the -33A allele decreases promoter activity. Interestingly, it has been found that in CAD patients homozygous -33G allele soluble thrombomodulin levels increased with the extent

of CAD. In CAD patients who were homozygous or heterozygous for the -33A allele, levels of soluble thrombomodulin did not change with the extent of vessel disease.

Poor outcome from infection has been shown to be highly heritable. Genotype has been shown to contribute substantially to outcome in sepsis (25, 27, 34). The genetic contribution to death from sepsis exceeds the inherited genetic contribution to cancer risk of death by many fold and even exceeds the genetic contribution to cardiovascular disease risk (39). Genetic polymorphisms in molecules of the protein C pathway may cause inter-individual variation in the response to infection, and may be predictive of patients' outcome from sepsis. If our current findings hold up in larger populations then we could conclude that the haplotype clade marked by the 5110A, 5318C, and 6235A alleles of the thrombomodulin gene is a marker of improved outcome in critically ill patients. Conceivably this knowledge would be helpful in identifying critically ill patients who may benefit from directed therapy, specifically patients who did not carry a haplotype from within the clade marked by 5110A, 5318C, and 6235A. Clinicians would use this knowledge to identify critically ill patients at risk for adverse outcome to design patient-tailored therapy based on genotype.

As recombinant human Activated Protein C (rhAPC) and other anti-coagulatory treatments become standard care in sepsis, it is essential to test genetic variants of coagulation system genes for association to outcome in sepsis in order to incorporate genotype into the design of patient-tailored therapy. Currently, rhAPC is indicated only for patients with severe sepsis (APACHE II $\geq$ 25). If patients at risk by genotype for poor outcome from sepsis could be identified prospectively, rhAPC could be administered to these patients earlier and potentially lower mortality from sepsis even further.

Drug companies could use knowledge of genetic risk factors for poor outcome from sepsis to target their randomized control trials to patients who would benefit most from drug therapies with few side effects based on their genotype. Our association of haplotypes of thrombomodulin with poor outcome in critically ill patients would be particularly useful in trials of drugs that modify coagulation and fibrinolysis such as activated protein C, protein C concentrate, tissue factor pathway inhibitor (TFPI), heparin, tissue plasminogen activator and other drugs in development may be more beneficial in patients identified to be at increased risk of death and organ dysfunction.

Examples:

252 consecutive critically ill patients admitted to the ICU of St. Paul's Hospital were screened for inclusion. Of these, 223 Caucasian patients were successfully genotyped and make up the cohort of this study.

Example 1
Haplotype clade deduction

We were able to infer haplotypes from complete sequencing of THBD for 23 Caucasians in the Coriell Cell Repository (37) using PHASE software (40), and identified two major haplotype clades using MEGA2 software (20) (Figures 1 and 2). These 5 clades could be resolved by genotyping three htSNPs: G5110A, A5318C and A6235G, in our 223 patient cohort. The 5110G/5318A/6235A (G/A/A) haplotype clade occurred with a frequency of 36.3%, the A/A/A haplotype clade occurred with a frequency of 22.4%, and a/A/G haplotype clade occurred with a frequency of 21.5%, the A/C/A haplotype clade occurred with a frequency of 18.4%, and the G/A/G haplotype clade occurred with a frequency of 1.3%. The genotypes of all three htSNPs were similar to frequencies deduced from other available Caucasian data(37) and were in Hardy-Weinberg equilibrium (Table 2) (14).

For the 223 successfully genotyped individuals of the cohort of Caucasian patients who had at least 2 of 4 SIRS criteria, no haplotype clade of THBD was significantly associated with a difference in age, gender or severity of illness at the time of admission to the study (as estimated by the APACHE II score) (Table 2). By chance, the A/C/A haplotype clade was associated with a higher proportion of surgical diagnoses for admission to the ICU (Table 2).

Example 2
Haplotype patient outcome

Upon preliminary analysis by ANOVA, the A/C/A haplytype clade appeared to be associated with a lower rate of 28-day mortality than the G/A/A, A/A/A, G/A/G, and A/A/G haplotype clades (Figure 3). This trend was stronger in patients who had sepsis or septic shock at the time they were admitted to the study (Figure 4). We subsequently chose to compare the 4 haplotype clades which were associated with increased rates of 28-day mortality as a group to the A/C/A haplotype clade. Further analysis was limited to the 130 patients who had sepsis or septic shock at the time they were admitted to the study. The average APACHE II score of these patients was 21.4 ± 7.9. There was no difference between clades in the proportion of medical vs. surgical diagnoses in this subgroup of patients.

In patients who had sepsis or septic shock at the time they were admitted to the study, the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with significantly greater 28-day mortality than the A/C/A haplotype clade (p=0.03) (Figure 5a). Kaplan-Meier analysis of 28-day mortality verified that the G/A/A, A/A/A, G/A/G and A/A/G haplotype clades were significantly associated

with increased rates of mortality over the entire 28-day observation period (p<0-.03) (Figure 5b). A Cox multiple regression model demonstrated that the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was an independent predictor of mortality after adjusting for other predictors of survival (age, sex, medical vs surgical diagnosis at admission) (p<0.03) (Table 4).

The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with a more vigorous inflammatory response. In our entire 223 patient cohort, the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer DAF of 4 of 4 (20.6 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.1 days for the A/C/A clade, p=0.05), 3 of 4 (20.3 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 22.7 days for the A/C/A clade, p=0.06) and 2 of 4 SIRS criteria (19.9 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 22.4 days for the A/C/A clade, p=0.05). In the subgroup of 130 patients who had sepsis or septic shock upon admission to the study the G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was even more strongly associated with fewer DAF of 4 of 4 (20.0 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.9 days for the A/C/A clade, p=0.01), 3 of 4 (19.7 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 23.1 days for the A/C/A clade, p=0.02) and 2 of 4 SIRS criteria (19.1 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs 23.0 days for the A/C/A clade, p=0.01).

The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer days alive and free of multiple-system organ failure. The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was significantly associated with fewer DAF of cardiovascular failure (p=0.02), and the need for more cardiovascular support as measured by fewer DAF of vasopressors (p=0.03) (Figure 6). The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was associated with fewer DAF of respiratory failure (p=0.02) and fewer DAF of ventilation (p=0.008) (Figure 7). The G/A/A, A/A/A, G/A/G and A/A/G haplotype clade group was also associated with fewer DAF of hematologic system failure (23.8 days for the G/A/A, A/A/A, G/A/G and A/A/G clades vs. 26.5 days for the A/C/A clade, p=0.04) fewer DAF of neurologic dysfunction (18.4 for the G/A/A, A/A/A, G/A/G and A/A/G clade vs. 22.1 days for the A/C/A clade, p=0.02), and fewer DAF of hepatic dysfunction (18.1 days for the G/A/A, A/A/A, G/A/G and A/A/G clade vs. 21.6 days for the A/C/A clade, p=0.04).

When analyzed individually, there was no significant association between the htSNPs G5110A, A5318C, or A6235G and 28-day mortality or multiple system organ failure.

References:

1. **Akey, J., L. Jin, and M. Xiong.** 2001. Haploty[es vs single marker linkage disequilibrium tests: what do we gain? Eur. J. Hum Genet **9**:291-300.
2. **Anonymous.** 1992. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis.[comment]. Critical Care Medicine **20**:864-74.
3. **Anonymous.** 2000. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med **342**:1301-8.
4. **Bernard, G. R., A. Artigas, K. L. Brigham, and e. al.** 1994. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med **149**:818-24.
5. **Beutler, B.** 2001. Sepsis begins at the interface of pathogen and host. Biochem Soc Trans **29**:853-9.
6. **Bochud, P. Y., T. R. Hawn, and A. Aderem.** 2003. Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. J Immunol **170**:3451-4.
7. **Conway, E. M., M. Van de Wouwer, S. Pollefeyt, K. Jurk, H. Van Aken, A. De Vriese, J. I. Weitz, H. Weiler, P. W. Hellings, P. Schaeffer, J. M. Herbert, D. Collen, and G. Theilmeier.** 2002. The lectin-like domain of thrombomodulin confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways. J Exp Med **196**:565-77.
8. **Doggen, C. J., G. Kunz, F. R. Rosendaal, D. A. Lane, H. L. Vos, P. J. Stubbs, V. Manger Cats, and H. Ireland.** 1998. A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. Thromb Haemost **80**:743-8.
9. **Esmon, C. T., N. L. Esmon, and K. W. Harris.** 1982. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. J Biol Chem **257**:7944-7.
10. **Esmon, C. T., and W. G. Owen.** 1981. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci U S A **78**:2249-52.
11. **Esmon, N. L., R. C. Carroll, and C. T. Esmon.** 1983. Thrombomodulin blocks the ability of thrombin to activate platelets. J Biol Chem **258**:12238-42.
12. **Gabriel, S. B., S. F. Schaffner, H. Nguyen, and e. al.** 2002. The structure of haplotype blocks in the human genome. Science **296**:2225-9.
13. **Guo, S. W., and E. A. Thompson.** 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics **48**:361-72.
14. **Guo, S. W., and E. A. Thompson.** 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics **48**:361-72.

15. **Ireland, H., G. Kunz, K. Kyriakoulis, P. J. Stubbs, and D. A. Lane.** 1997. Thrombomodulin gene mutations associated with myocardial infarction. Circulation **96**:15-8.
16. **Johnson, G. C., L. Esposito, B. J. Barratt, and e. al.** 2001. Haplotype tagging for the identification of common disease genes. Nat Genet **29**:233-7.
17. **Kang, T. J., S. B. Lee, and G. T. Chae.** 2002. A polymorphism in the toll-like receptor 2 is associated with IL-12 production from monocyte in lepromatous leprosy. Cytokine **20**:56-62.
18. **Knaus, W. A., D. P. Wagner, E. A. Draper, J. E. Zimmerman, M. Bergner, P. G. Bastos, C. A. Sirio, D. J. Murphy, T. Lotring, A. Damiano, and et al.** 1991. The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults
Utilizing findings from the APACHE III research to develop operational information system for the ICU--the APACHE III ICU Management System
APACHE II: a severity of disease classification system. Chest **100**:1619-36.
19. **Krishnaswamy, S., E. B. Williams, and K. G. Mann.** 1986. The binding of activated protein C to factors V and Va. J Biol Chem **261**:9684-93.
20. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics anslysis software. Bioinformatics **17**:1244-5.
21. **Kunz, G., A. K. Ohlin, A. Adami, B. Zoller, P. Svensson, and D. A. Lane.** 2002. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. Blood **99**:3646-53.
22. **Lignell, A., A. Siegbahn, M. Stridsberg, K. Pauksen, R. Gedeborg, and J. Sjolin.** 2003. Low utilisation of unactivated protein C in a patient with meningococcal septic shock and disseminated intravascular coagulation. Acta Anaesthesiol Scand **47**:897-900.
23. **Livak, K. J.** 1999. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal **14**:143-9.
24. **Lorenz, E., J. P. Mira, K. L. Cornish, N. C. Arbour, and D. A. Schwartz.** 2000. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. Infect Immun **68**:6398-401.
25. **Majetschak, M., S. Flohe, U. Obertacke, and e. al.** 1999. Relation of aTNF gene polymorphism to severe sepsis in trauma patients. Ann Surg **230**:207-14.
26. **Mann, K. G., M. F. Hockin, K. J. Begin, and M. Kalafatis.** 1997. Activated protein C cleavage of factor Va leads to dissociation of the A2 domain. J Biol Chem **272**:20678-83.
27. **Mira, J. P., A. Cariou, F. Grall, and e. al.** 1999. Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study [see comments]. JAMA **282**:561-8.
28. **Moore, K. L., S. P. Andreoli, N. L. Esmon, C. T. Esmon, and N. U. Bang.** 1987. Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. J Clin Invest **79**:124-30.
29. **Nakazawa, F., T. Koyama, A. Shibamiya, and S. Hirosawa.** 2002. Characterization of thrombomodulin gene mutations of the 5'-regulatory region. Atherosclerosis **164**:385-7.

30. **O'Brien, L. M., M. Mastri, and P. J. Fay.** 2000. Regulation of factor VIIIa by human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. Blood **95:**1714-20.

31. **Odegaard, B., and K. Mann.** 1987. Proteolysis of factor Va by factor Xa and activated protein C. J Biol Chem **262:**11233-8.

32. **Ogawa, S., H. Gerlach, C. Esposito, A. Pasagian-Macaulay, J. Brett, and D. Stern.** 1990. Hypoxia modulates the barrier and coagulant function of cultured bovine endothelium. Increased monolayer permeability and induction of procoagulant properties. J Clin Invest **85:**1090-8.

33. **Ohnishi, Y., T. Tanaka, R. Yamada, K. Suematsu, M. Minami, K. Fujii, N. Hoki, K. Kodama, S. Nagata, T. Hayashi, N. Kinoshita, H. Sato, T. Kuzuya, H. Takeda, M. Hori, and Y. Nakamura.** 2000. Identification of 187 single nucleotide polymorphisms (SNPs) among 41 candidate genes for ischemic heart disease in the Japanese population. Hum Genet **106:**288-92.

34. **Read, R. C., N. J. Camp, F. S. di Giovine, and e. al.** 2000. An interleukin-1 genoty[e is associated with fatal outcome of meningococcal disease. J Infect Dis **182:**1557-60.

35. **Rieder, M. J., Armel,T.Z., Carrington,D.P., Chung,M.-W., Lee,K.L., Ozuna,M., Poel,C.L., Toth,E.J., Yi,Q. and Nickerson,D.A.** 26-March-2002 2002, posting date. AF495471. Homo sapiens thrombomodulin (THBD) gene, complete cds. SeattleSNPs. NHLBI HL66682 Program for Genomic Applications, UW-FHCRC. [Online.]

36. **Russell, J. A., J. Singer, G. R. Bernard, A. Wheeler, W. Fulkerson, L. Hudson, R. Schein, W. Summer, P. Wright, and K. R. Walley.** 2000. Changing pattern of organ dysfunction in early human sepsis is related to mortality. Crit Care Med **28:**3405-11.

37. **SeattleSNPs** 2003, posting date. Thrombomodulin. SeattleSNPs. NHLBI Programs for Genomic Applications. UW-FHCRC. [Online.]

38. **Sibbald, W. J., and J. L. Vincent.** 1995. Round table conference on clinical trials for the treatment of sepsis. Brussells, March 12-14, 1994. Intensive Care Med **21:**184-9.

39. **Sorensen, T. I., G. G. Nielsen, P. K. Andersen, and T. W. Teasdale.** 1988. Genetic and environmental influences on premature death in adult adoptees. N Engl J Med **318:**727-32.

40. **Stephens, M., N. J. Smith, and P. Donnelly.** 2001. A new statistical method for haplotype reconstruction from population data. Am J Respir Crit Care Med **68:**978-89.

41. **Templeton, A. R., K. M. Weiss, D. A. Nickerson, E. Boerwinkle, and C. F. Sing.** 2000. Cladistic structure within the human lipoprotein lipase gene and its implications for phenoty[ic association studies. Genetics **156:**1259-75.

42. **Weiler-Guettler, H., P. D. Christie, D. L. Beeler, A. M. Healy, W. W. Hancock, H. Rayburn, J. M. Edelberg, and R. D. Rosenberg.** 1998. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest **101:**1983-91.

43. **Zhang, K., P. Calabrese, M. Nortborg, and F. Sun.** 2002. Haplotype block structure and its applications to association studies: power and study designs. Am J. Hum. Genet **71**:1386-94.

TABLE 1
Brussels Organ Dysfunction Scoring System

ORGANS	Free of Organ Dysfunction		Clinically Significant Organ Dysfunction		
	Normal	Mild	Moderate	Severe	Extreme
Cardiovascular Systolic BP (mmHg)	>90	≤90 Responsive to fluid	≤90 Unresponsive to fluid	≤90 plus pH ≤7.3	≤90 plus pH ≤7.2
Pulmonary P_aO_2/F_IO_2 (mmHg)	>400	400-301	300-201 Acute lung injury	200-101 ARDS	≤100 Severe ARDS
Renal Creatinine (mg/dL)	<1.5	1.5-1.9	2.0-3.4	3.5-4.9	≥5.0
Hepatic Bilirubin (mg/dL)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	≥12
Hematologic Platelets ($x10^5/mm^3$)	>120	120-81	80-51	50-21	≤20
Neurologic (Glascow Score)	15	14-13	12-10	9-6	≤5
Round Table Conference on Clinical Trials for the Treatment of Sepsis Brussels, March 12-14, 1994 (38).					

TABLE 2. Genotype Frequencies and Allele Frequencies for three htSNPs of thrombomodulin in a Cohort of 223 Critically Ill Adults who had SIRS

	Genotype Frequencies			Allele Frequencies		p *
	GG	GA	AA	G	A	
G5110A	17%	42%	41%	38%	62%	0.119
	AA	AC	CC	A	C	
A5318C	67%	29%	4%	82%	18%	0.514
	AA	AG	GG	A	G	
A6235G	62%	31%	7%	77%	23%	0.086

* exact test of Guo and Thompson to test for Hardy-Weinberg equilibrium (13)

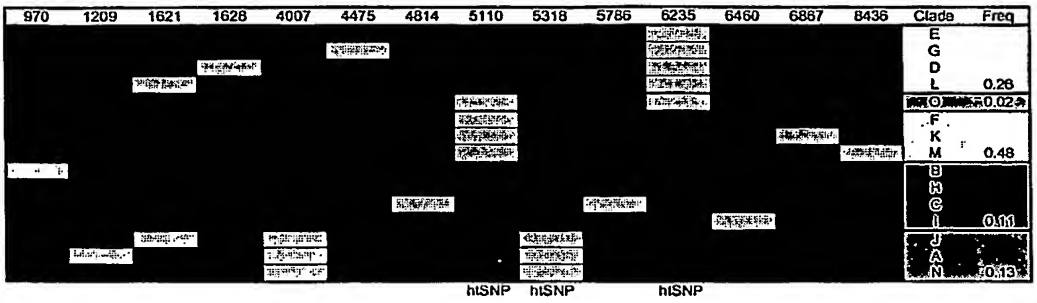
TABLE 3. Baseline Characteristics of 223 critically ill patients with SIRS by thrombomodulin haplotype clade

Haplotype Clade	Frequency	Mean Age	Gender (% Male)	Diagnosis for admission (% Surgical)	Mean APACHE II
G/A/A	36%	59	60%	26%	18
A/C/A	18%	59	61%	44%	19
A/A/A	22%	59	69%	23%	20
G/A/G	1%	69	50%	17%	19
A/A/G	22%	61	68%	33%	20
p		NS	NS	0.02	NS

TABLE 4. Cox Proportional Hazard Analysis – Hazard Ratios for Mortality

Covariate	Hazard Ratio	95% CI	p
Female sex	0.63	0.41-0.98	0.04
Age	1.00	0.99-1.02	0.45
Surgical Diagnosis	0.77	0.50-1.17	0.21
G/A/A, A/A/A, G/A/G, or A/A/G	1.95	1.05-3.57	0.03

Figure 1. Haplotype structure of the thrombomodulin gene in Caucasians



5

Figure 2. Unrooted phylogenetic tree of thrombomodulin haplotypes in Caucasians.

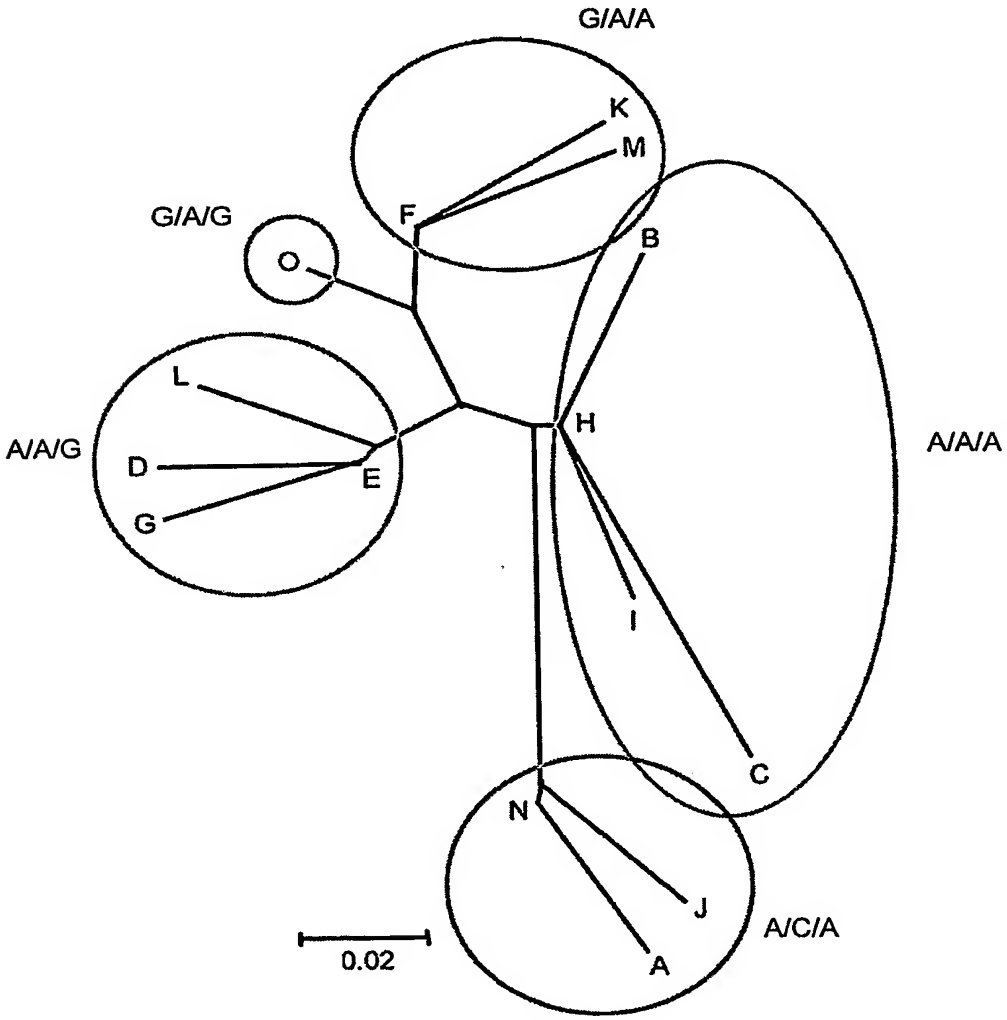


Figure 3. 28-day Mortality in 223 critically ill patients with SIRS by haplotype clade

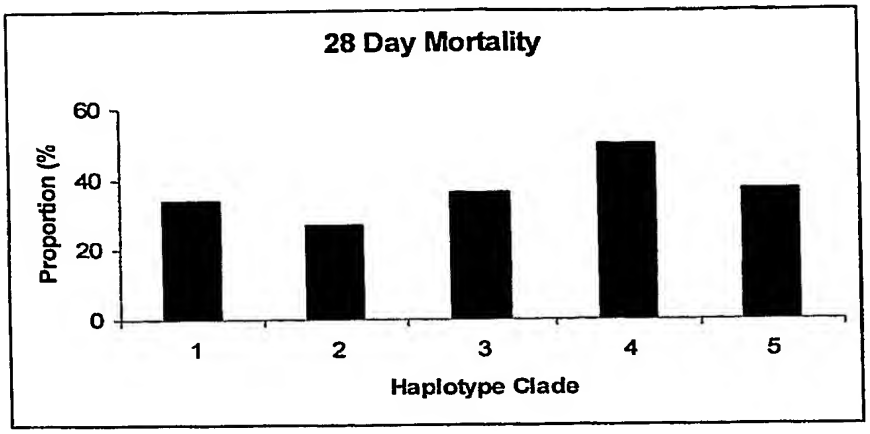


Figure 4. 28 day Mortality in 130 critically ill patients with sepsis or septic shock on day one of observation.

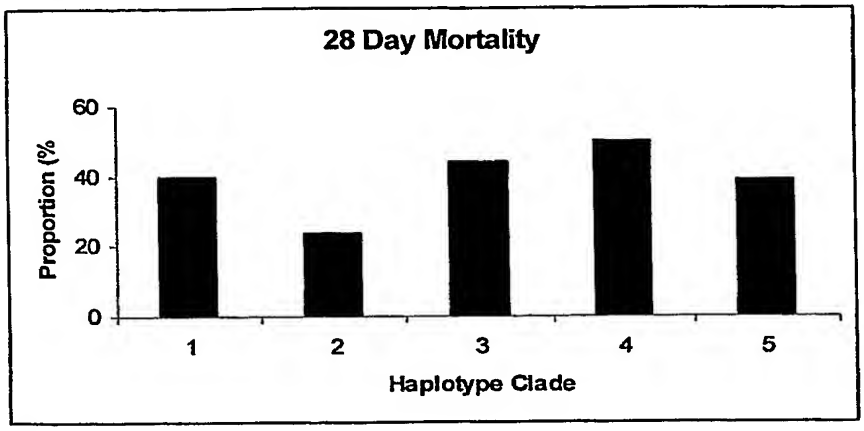


Figure 5a. 28 day Mortality in 130 patients with sepsis or septic shock on day one of the study.

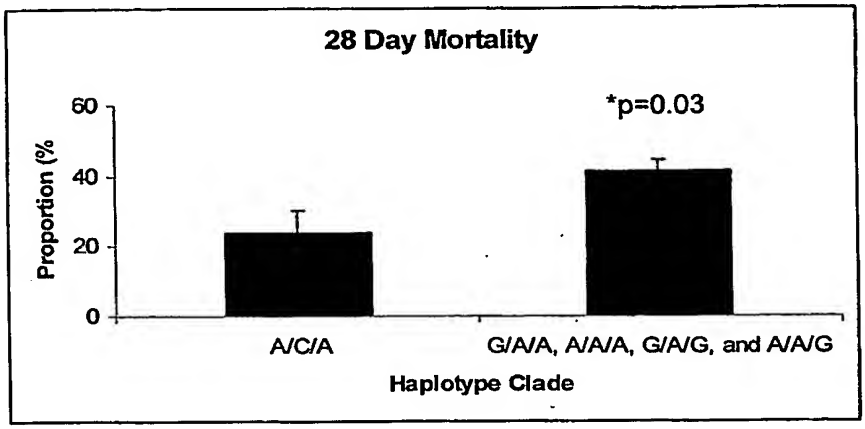


Figure 5b. Kaplan-Meier survival analysis in 130 patients with sepsis or septic shock on day one of the study.

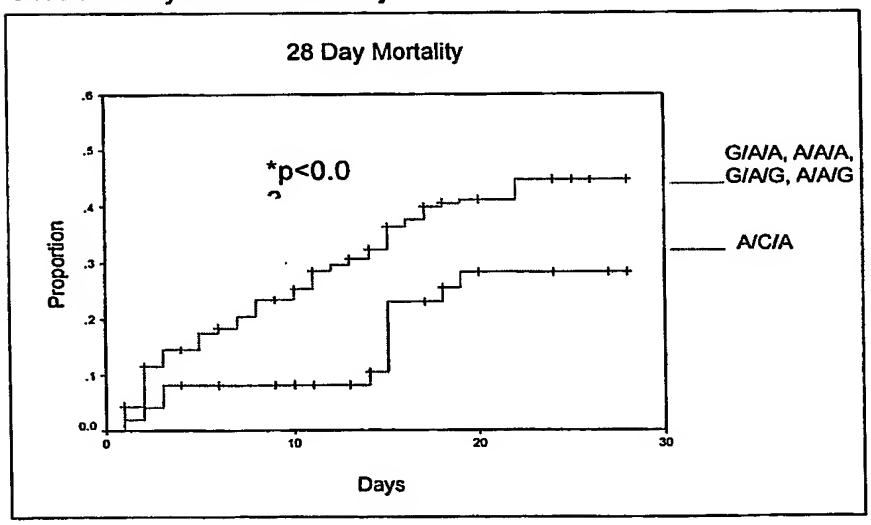


Figure 6. Days alive and free of Cardiovascular dysfunction in 130 patients with sepsis or septic shock on day one of the study.

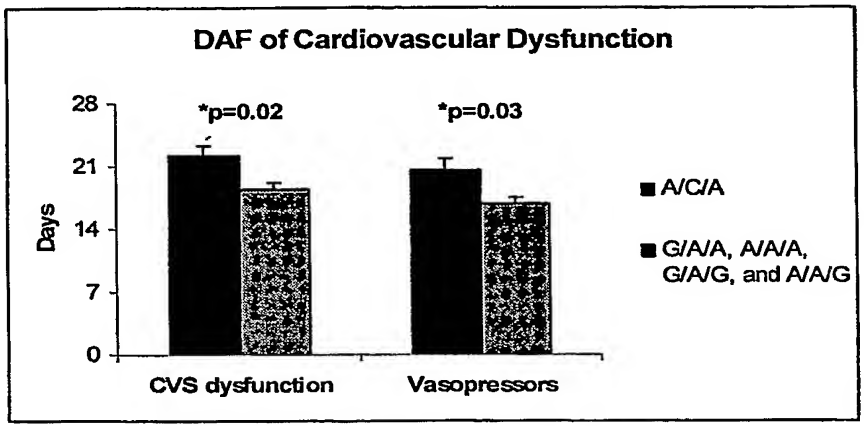
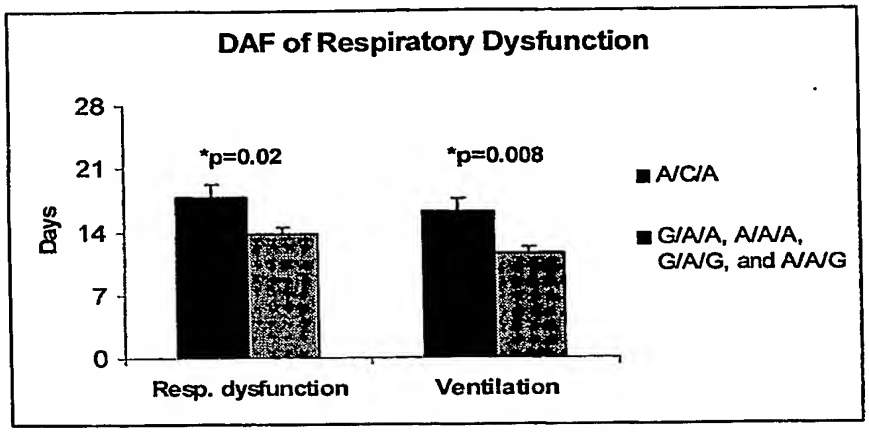


Figure 7. Days alive and free of Respiratory dysfunction in 130 critically ill patients with sepsis or septic shock on day one of study.



5

SEQ ID NO.1

G5110A
CAGATTCCCAGAGCAAAATAATTTTAAACAAAGGTTGAGATGTAAAAGGT[G/A]TTAA
ATTGATGTTGCTGGACTGTCATAGAAATTACACCCAAAGAGGTATT

A5318C
TTACTTATTTTTGACAGTGTTGAAAATGTTCAGAAGGTTGCTCTAGATTG[A/C]GAGA
AGAGACAAACACCTCCCAGGAGACAGTTCAAGAAAGCTTCAAACTG

A6235G
TGAGATGCATGGAGGGCTGCCCTGTACCCCAGCACTTGTGTTGTCTGGTG[A/G]TG
GCACCATCTCTGATTTTCAAAGCTTTTTCCAGAGGCTATTATTTTCAC

SEQ ID. NO. 2

AF495471.1 Thrombomodulin (human)

```
   1 atctgcacct cctcatatag ggttgatcca agtttcacag acatcactga gttcttagtg
  61 gactcagcta ttggggctgt tctcacactt ttttttctt tgcaagaatc agcaatgggt
 121 gcaagtggac ctgtgtagga cgtccagtga aacattgtgt tggtgaatca gctagaatcc
 181 atccaagaac tcagccagcc tggtgtgggg tgagatctga tccttgaatg tccctcagtg
 241 gcttttaggg ctggcaggtt cagaagggcc ctctcatcac ccccccaggg cctcattcct
 301 tgtttaacac tttgctatca cagtcttgaa tccttgtaat tgaacaatgg accccacatt
 361 ttcactttgc actggtttct gattctgtaa ccgatcctgt ccccctctct tgtctcattc
 421 actctgggaa ttgtccccac attctgagac ctttcagcag tgccccaacg aggttcctgc
 481 ccttatctga agctccaccc tcaccccccat ggcggcaccg caggcagccc tgcttttgcg
 541 tcccgcgtag gcaggctgtg caccggagtc acgacccccc gattcagcct aggcagccac
 601 agcttgactg ctcccgccgg acaagcccta ctgtgctatc tgccgctctt cccttcctct
 661 tcccaggggg tccgcgtcag gggaggcgca gctgtgtgca ttccgggagc ttcagacccc
 721 cgtgtccagc agctccttcg tttcctgggt gctggggcgg ccttcccagc gaagagctca
 781 actcagcggg acgtttggag gctctctgcc ccaaggcgct ggggagtgtg cggcgggaca
 841 gtcgtgcttg ccttttcac tttcagagtg tccacgcccc acccgtttgg tcactgcagg
 901 tcagtccagt ccagcccggc ccaccccacc ggtgcgtgtc tgtcgcacgt ggcagacgcc
 961 atactctctg ttcttgttta aagcccagga tctactgggc cctggaggca agaggtgaac
1021 gcagcggaat ccacgctgag ctgcccggga acggagcttc caaccccaga aggaggactc
1081 tgtgctccta caccttaacc ctttttagcc cgaaacttct ccaacttcct tggctttgtt
1141 tagagctcga cagcgccgcc ccctggcgct cgttgtgagg acagtagagg agagaggcaa
1201 gggtgttttt aaacagtttg cctctcacca ttatgggggc gacccgaggg ggagacccac
1261 tcttccgcat tcccggtaag tgaaccaccg gaagaggtcg aaagtgacgg attcccatgt
1321 cctcctccag ccccccccc accctgccca tccacaggac ggtggctctt cagtgccctt
1381 tgccgagcaa gtggcgtttc tatgcacgtg ggtatcaatt cggactctgg acgaaatgga
1441 aacctcctta gccgacccgg gtgggatcag ctgggatcct gcgcgctccc ctgggggttt
1501 gccagccact ctgttggggt gcaagaagca ccatccttcg gaagctgggc cgaaactggc
1561 caggctgact cgctcccacg cgcccgcccc tacccggcgc cgcagcaatt cacctgccac
1621 cgcctctgag ccgggtccgg acttcggcgc cctgacagtg tccccgcgac ttccccaccc
1681 gatgagatgg ggtctggcgt tggccagtgc gtgtccaggg actcgcgggt ccctggccag
1741 ccatggggca gagggcgctg gtgttaggcc agtcttcccc accctgcccc gtcaccccag
1801 ccacacccac tgtcctgtga ggccaagcgc gctccgctgg tttcctgagc caggcacctt
1861 ggccgcggac aggatccagc tgtctctcct tgcgatcctg tcttcgggga agtccacgtc
1921 ctaggcaggt cctcccaaag tgcccttggt gccgatcacc cctcccagcg tcttgcaggt
1981 cctgtgcacc acctcccccа ctccccattc aaagccctct tctctgaagt ctccggttcc
2041 cagagctctt gcaatccagg ctttccttgg aagtggctgt aacatgtatg aaaagaaaga
2101 aaggaggacc aagagatgaa agagggctgc acgcgtgggg gcccgagtgg tgggcgggga
2161 cagtcgtctt gttacagggg tgctggcctt ccctggcgcc tgcccctgtc ggccccgccc
2221 gagaacctcc ctgcgccagg gcagggttta ctcatcccgg cgaggtgatc ccatgcgcga
2281 gggcgggcgc aagggcggcc agagaaccca gcaatccgag tatgcggcat cagcccttcc
```

```
2341 caccaggcac ttccttcctt ttcccgaacg tccagggagg gagggccggg cacttataaa
2401 ctcgagccct ggccgatccg catgtcagag gctgcctcgc aggggctgcg cgcagcggca
2461 agaagtgtct gggctgggac ggacaggaga ggctgtcgcc atcggcgtcc tgtgcccctc
2521 tgctccggca cggccctgtc gcagtgcccg cgctttcccc ggcgcctgca cgcggcgcgc
2581 ctgggtaaca tgcttggggt cctggtcctt ggcgcgctgg ccctggccgg cctggggttc
2641 cccgcacccg cagagccgca gccgggtggc agccagtgcg tcgagcacga ctgcttcgcg
2701 ctctacccgg gccccgcgac cttcctcaat gccagtcaga tctgcgacgg actgcggggc
2761 cacctaatga cagtgcgctc ctcggtggct gccgatgtca tttccttgct actgaacggc
2821 gacggcggcg ttggccgccg gcgcctctgg atcggcctgc agctgccacc cggctgcggc
2881 gaccccaagc gcctcgggcc cctgcgcggc ttccagtggg ttacgggaga caacaacacc
2941 agctatagca ggtgggcacg gctcgacctc aatggggctc ccctctgcgg cccgttgtgc
3001 gtcgctgtct ccgctgctga ggccactgtg cccagcgagc cgatctggga ggagcagcag
3061 tgcgaagtga aggccgatgg cttcctctgc gagttccact tcccagccac ctgcaggcca
3121 ctggctgtgg agcccggcgc cgcggctgcc gccgtctcga tcacctacgg caccccgttc
3181 gcggcccgcg gagcggactt ccaggcgctg ccggtgggca gctccgccgc ggtggctccc
3241 ctcggcttac agctaatgtg caccgcgccg cccggagcgg tccaggggca ctgggccagg
3301 gaggcgccgg gcgcttggga ctgcagcgtg gagaacggcg gctgcgagca cgcgtgcaat
3361 gcgatccctg gggctccccg ctgccagtgc ccagccggcg ccgccctgca ggcagacggg
3421 cgctcctgca ccgcatccgc gacgcagtcc tgcaacgacc tctgcgagca cttctgcgtt
3481 cccaaccccg accagccggg ctcctactcg tgcatgtgcg agaccggcta ccggctggcg
3541 gccgaccaac accggtgcga ggacgtggat gactgcatac tggagcccag tccgtgtccg
3601 cagcgctgtg tcaacacaca gggtggcttc gagtgccact gctaccctaa ctacgacctg
3661 gtggacggcg agtgtgtgga gcccgtggac ccgtgcttca gagccaactg cgagtaccag
3721 tgccagcccc tgaaccaaac tagctacctc tgcgtctgcg ccgagggctt cgcgcccatt
3781 ccccacgagc cgcacaggtg ccagatgttt tgcaaccaga ctgcctgtcc agccgactgc
3841 gaccccaaca cccaggctag ctgtgagtgc cctgaaggct acatcctgga cgacggtttc
3901 atctgcacgg acatcgacga gtgcgaaaac ggcggcttct gctccggggt gtgccacaac
3961 ctccccggta ccttcgagtg catctgcggg cccgactcgg cccttgcccg ccacattggc
4021 accgactgtg actccggcaa ggtggacggt ggcgacagcg gctctggcga gccccgccc
4081 agcccgacgc ccggctccac cttgactcct ccggccgtgg ggctcgtgca ttcgggcttg
4141 ctcataggca tctccatcgc gagcctgtgc ctggtggtgg cgcttttggc gctcctctgc
4201 cacctgcgca agaagcaggg cgccgccagg gccaagatgg agtacaagtg cgcggccctc
4261 tccaaggagg tagtgctgca gcacgtgcgg accgagcgga cgccgcagag actctgagcg
4321 gcctccgtcc aggagcctgg ctccgtccag gagcctgtgc ctcctcaccc ccagctttgc
4381 taccaaagca ccttagctgg cattacagct ggagaagacc ctccccgcac cccccaagct
4441 gttttcttct attccatggc taactggcga gggggtgatt agagggagga gaatgagcct
4501 cggcctcttc cgtgacgtca ctggaccact gggcaatgat ggcaattttg taacgaagac
4561 acagactgcg atttgtccca ggtcctcact accgggcgca ggagggtgag cgttattggt
4621 cggcagcctt ctgggcagac cttgacctcg tgggctaggg atgactaaaa tatttatttt
4681 ttttaagtat ttaggttttt gtttgtttcc tttgttctta cctgtatgtc tccagtatcc
4741 actttgcaca gctctccggt ctctctctct ctacaaactc ccacttgtca tgtgacaggt
4801 aaactatctt ggtgaatttt tttttcctag ccctctcaca tttatgaagc aagccccact
4861 tattccccat tcttcctagt tttctcctcc caggaactgg gccaactcac ctgagtcacc
4921 ctacctgtgc ctgaccctac ttcttttgct cttagctgtc tgctcagaca gaacccctac
4981 atgaaacaga aacaaaaaca ctaaaaataa aaatggccat ttgctttttc accagatttg
5041 ctaatttatc ctgaaatttc agattcccag agcaaaataa ttttaaacaa aggttgagat
5101 gtaaaaggta ttaaattgat gttgctggac tgtcatagaa attacaccca aagaggtatt
5161 tatctttact tttaaacagt gagcctgaat tttgttgctg ttttgatttg tactgaaaaa
5221 tggtaattgt tgctaatctt cttatgcaat ttccttttt gttattatta cttatttttg
5281 acagtgttga aaatgttcag aaggttgctc tagattgaga gaagagacaa acacctccca
5341 ggagacagtt caagaaagct tcaaactgca tgattcatgc caattagcaa ttgactgtca
5401 ctgttccttg tcactggtag accaaaataa aaccagctct actggtcttg tggaattggg
5461 agcttgggaa tggatcctgg aggatgccca attagggcct agccttaatc aggtcctcag
5521 agaatttcta ccatttcaga gaggccttt ggaatgtggc ccctgaacaa gaattggaag
5581 ctgccctgcc catgggagct ggttagaaat gcagaatcct aggctccacc ccatccagtt
5641 catgagaatc tatatttaac aagatctgca gggggtgtgt ctgctcagta atttgaggac
5701 aaccattcca gactgcttcc aattttctgg aatacatgaa atatagatca gttataagta
5761 gcaggccaag tcaggccctt attttcaaga aactgaggaa ttttctttgt gtagctttgc
5821 tctttggtag aaaaggctag gtacacagct ctagacactg ccacacaggg tctgcaaggt
5881 ctttggttca gctaagctag gaatgaaatc ctgcttcagt gtatggaaat aaatgtatca
5941 tagaaatgta acttttgtaa gacaaaggtt ttcctcttct attttgtaaa ctcaaaatat
6001 ttgtacatag ttatttattt attggagata atctagaaca caggcaaaat ccttgcttat
6061 gacatcactt gtacaaaata aacaaataac aatgtgctct cgggttgtgt gtctgttcac
```

```
6121 ttttcctccc tcagtgccct cattttatgt cattaaatgg ggctcacaaa ccatgcaaat
6181 gctatgagat gcatggaggg ctgccctgta ccccagcact tgtgttgtct ggtgatggca
6241 ccatctctga ttttcaaagc tttttccaga ggctattatt ttcactgtag aatgatttca
6301 tgctatctct gtgtgcacaa atatttattt tctttctgta accataacaa cttcatatat
6361 gaggacttgt gtctctgtgc ttttaaatgc ataaatgcat tataggatca tttgttggaa
6421 tgaattaaat aaacccttcc tggggcatct ggcgaatccc agctgtgtgt ccggtgtatg
6481 gtttggcatt atctcctctg cgagatatcc aaattcactg tagtcatgaa gggtctcagt
6541 ttgtggctct cattcaaata ttcatttcta aacgtctcat ccagtatgaa atcattctca
6601 tctcttttgg agattaacaa catcatcttt tcaatgcaca cgtttcttgg gctcactttt
6661 ctaaggtggt agggctggct gaatgcaata tgcagggctc ggaaagattt tttaaagaag
6721 aaattaaaag caagtagagt ccaggcaaat attcagatgc tttatatgtc tggataatgc
6781 tgaactcatg agttttagtt tgactgatta ttgtgaagac cgggttggag attttgacat
6841 ccatcgcaga agaagtaatg gctttagtgt gtgtgtgtgt gtgtgctggg gaagctccat
6901 gcacagtgcc ctatggagat aacaagctga gccatgctcc ccctaagtag cagactaagt
6961 ctttgtgaag gaagagctac acaaatgggg gcaggacagg tgcagataaa tggggctggg
7021 agaccagagg agacagtgac accttatagt tcgccccctg ttacccagcc ttctgtttgt
7081 caaaagagtc tgctcccagt cactgtcaaa ctgacttgta gggcctcatt gcgttaggat
7141 ttcttcttat tccagaagag gggcattttc ttaaggaaca ctggaagacc aaaacacact
7201 ttcaaaacct agaggcaaaa acccttcatg cagcacttgg gccccaggac attagttgtg
7261 cggggccctg agcttccctg tcctcctcac ttcctgctgc ctgggggatc agcagttctg
7321 tttataggtc tcatctgaac ttgagattct caaaacgcta aatagccata gtgcctctca
7381 gggaaagata ccaggaccac ataaacaaat cagttagctt taaaaactat ccctgagcat
7441 ttaaaatcag gatagacctt gtgaaaccag agccatgggt caacctgtgt gatctctgct
7501 ttctgttcac atcattggac atccaggtct gagggagact cccagggacc agttgctggt
7561 gaaatttcat agcacaaaag tccggggcaa gaaagccaag gtggtatttc tggataagcc
7621 agcattcaag tttgtttgtt tgtttgtttg tttgtttttc ctagcctgct gttttaaagt
7681 aaacagaatg catttttta agtcaaatga ctttgttatt tttttttcc agttctcacc
7741 tatttcttag attagttcag caattattta ctgagcattt actctgtgcc tttcatagtg
7801 ataggcacaa tgacaagtcc ctaccatata agttagactc tggcagggga gaaagatgca
7861 aaacaactga tcacccccaa attgtactta acttagaaac agtgataagt gcaggggaag
7921 aaaagcacag cacactctga aaaggcgcac gaggaaggca ggatttagag tggaggacta
7981 gagggagctt cctggacaag ctgacactta acaccagacc tgaaggggaa ggaggggttt
8041 gtcaaatgca aactggaggg gaagcagtcc aggtgggaag gatcacacct gcaaaggccc
8101 tgtactggga agagccctgg tggagcggac tgggcatagt gaacaaggtg aggtgggctg
8161 caaggcagct gaagaggtgg aaagagagat acaagcagtg ggagatgact gtaggggctg
8221 taggtcaaag acactgaaaa aaagactgaa agagtgacat tgaaaaatgt tctgggtgca
8281 agtgggggca ctcaaggagt tttgatgaga gtgcactggg attcaattta tgtactgcat
8341 tgtttgggaa gataacaact acttctagat gtatttacat gtccctcttg ggcaggaacc
8401 tgcacaattt ccgctgtaag caccccgcag ggctgatatg tggtgtgaac agcatacaca
8461 cctgggtgtg accccagcct gaaacctgct ggtcacatgg ccacgggcac cacatgaccc
8521 ttcaaaggct gt
```